Improved Methods for Quantification of Human Immunodeficiency Virus Type 1 RNA and Hepatitis C Virus RNA in Blood Using Spin Column Technology and Chemiluminescent Assays of PCR Products

Hsiang Ju Lin*, Tawesak Tanwandee, and F. Blaine Hollinger
Division of Molecular Virology, Baylor College of Medicine, Houston, Texas

The quantification of human immunodeficiency virus type 1 (HIV-1) RNA or hepatitis C virus (HCV) RNA has been facilitated by adapting a spin column procedure for sample preparation and the use of chemiluminescent detection of polymerase chain reaction (PCR) products in microtiter plate format. All materials were commercially available and relatively inexpensive. By making a single dilution prior to amplification, concentrations of 500 copies to 2.5 million HIV-1 1 RNA copies per mL and 1,000 copies to 50 million HCV RNA copies per mL could be determined on 140-μL samples. Between-run imprecision employing the improved procedure for HIV-1 RNA was 23%. Correlation of HIV-1 RNA concentrations obtained using chemiluminescent detection with values obtained by colorimetric assay of PCR products was 0.98. Correlation of HCV RNA concentration determined by the spin column–chemiluminescent assay procedure with those obtained by branched DNA methodology was 0.91. Spin columns could be used with serum or plasma containing acid-citrate-dextrose or heparin anticoagulant, but heparinized samples required treatment with heparinase prior to amplification. ***J Med Virol 51:56–63, 1997.***

KEY WORDS: HIV-1 RNA; HCV RNA; sensitivity; anticoagulants; spin column technology

INTRODUCTION

The concentration of human immunodeficiency virus type 1 (HIV-1) RNA in the circulation is recognized as an important marker; it is essential for assessing the efficacy of treatment and is useful for predicting outcome [Wei et al., 1995; Mellors et al., 1995]. The quantification of HIV-1 RNA in serum or plasma has presented some difficulties. There is at least 10^{10} times more protein than HIV-1 RNA by weight, yet PCR-based assays require that the sample be deproteinized. Further, RNAses that are normally present in the sample must be inactivated. The procedures for purification of HIV-1 RNA require a high degree of technical skill and are time-consuming [Mulder et al., 1994; Lin et al., 1992]. The range of HIV-1 RNA copy numbers per mL extends from the low hundreds to over 10^7 [Piatak et al., 1993]; thus, short of making serial dilutions, a detection system with a broad dynamic range is called for.

Similar problems are encountered in the quantitative assay of hepatitis C virus (HCV) genomes which, like HIV-1, are composed of single strands of RNA. Extraction of HCV RNA is laborious, and some methods of extraction may be inefficient [Goergen et al., 1994; Nolte et al., 1994; Hsuih et al., 1996]. HCV RNA concentrations in clinical blood specimens may reach 10^8 copies per mL, and serial dilutions are usually performed [Gretch et al., 1994; Whitby and Garson, 1995; Roth et al., 1996].

We describe procedures that make use of microspin technology for the purification of viral RNA from plasma or serum, amplification by means of reverse transcription (RT)-polymerase chain reaction (PCR), and chemiluminescent assays for detection of PCR products. Spin column technology effects the separation of viral RNA from proteins and other blood components by means of a silica-based membrane that binds RNA and DNA. Elution is accomplished by passing a small volume of water through the membrane.

The chemiluminescent detection assay entails incorporation of a biotinylated primer into the PCR product, hybridization of RNA probe to denatured PCR products, binding of RNA-DNA hybrids to streptavidin-coated

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*Correspondence to: Dr. H.J. Lin, Division of Molecular Virology, Baylor College of Medicine, 1 Baylor Plaza, Houston, TX 77030.

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wells in microtiter plate format, and subsequent binding to an alkaline phosphatase–linked antibody directed at RNA-DNA hybrids. Chemiluminescence is produced when a substrate is hydrolyzed by the alkaline phosphatase. The murine monoclonal antibody is specific for the structure of heteroduplex but is not sequence-specific; it has virtually no reactivity with single-stranded RNA or DNA or with double-stranded DNA [Lazar, 1994].

MATERIALS AND METHODS

Blood Specimens

Clinical blood specimens were collected in standard 10-mL red, yellow and green top vacutainer tubes that contained, respectively, no anticoagulant, acid-citrate-dextrose (ACD), and sodium heparin anticoagulant (Bectin Dickinson, Rutherford, NJ). Aliquots of the cell-free fractions were stored at −80°C until tested.

Materials for Detection of PCR Products

All the materials mentioned in this section were obtained from Digene Diagnostics, Inc. (Silver Spring, MD). The HIV-1 probe and primer kit consisted of the primer pair SK38 and SK39 corresponding, respectively, to positions 1551–1578 and 1638–1665 in isolate HIV-1 BRU [Whetsell et al., 1992], an RNA probe that was a 244-nucleotide RNA transcript specific for HIV-1 isolate SF2 (GenBank accession number KO2007) in positions 1395–1638, and a positive assay control; the SK39 primer was biotinylated at the 5′ end. The HCV probe and primer kit contained a positive assay control, a primer pair homologous to sequences in the 5′-untranslated region, 5′GCACTCGCAAGCACCCTATC and 5′biotin-CTGTCTTCACGCAGAAAGCG that corresponded, respectively, to positions −28 to −47 and −284 to −265 in isolate HCV1 [Choo et al., 1991], and an RNA probe that was identical with the sequence of the amplified region. The negative assay control, probe diluent, sample diluent, denaturation reagent, wash buffer concentrate, and the alkaline phosphatase-linked antibody to RNA-DNA hybrids were components of the Sharp Signal System kit. Additional components of the chemiluminescent assay were LumiPhos® substrate for alkaline phosphatase and 96-well, streptavidin-coated microtiter plates for chemiluminescent detection.

Overview of Assay Protocols

For either assay, one 140-μL sample of each clinical specimen and one 140-μL sample each of five standards (ranging from 0 to 500,000 copies per mL) and an in-house low positive control are required. One spin column is used for each sample. RT-PCR assays are carried on the spin column eluates. Four RT-PCR assays are carried out on the eluate prepared from each clinical specimen: two with the undiluted eluate and two using either a 1:5 dilution for HIV-1 RNA or a 1:100 dilution for HCV RNA. The standards and positive control sample are amplified in duplicate from undiluted spin column eluates. Thus, 20 clinical specimens can be amplified in one batch and tested in 92 wells of a microtiter plate for chemiluminescent detection of PCR products, the remaining four wells being used for the negative and positive chemiluminescent assay controls.

The turnaround time for processing one batch of 20 clinical specimens is 2 days with an input of less than 5 hr on each day. Sample preparation and PCR assays are performed on the 1st day, and detection is carried out on the 2nd day.

HIV-1 RNA and HCV RNA Standards

HIV-1 RNA copy number standards were prepared by diluting a sample of strain HXB3 that had an HIV-1 particle count of over 10^{10} per mL [Layne et al., 1992] with ACD plasma seronegative for HIV-1, HIV-2, and other viruses that was obtained from The Blood Center, Houston. Calibration was performed against the HIV-1 RNA standards developed in an earlier study [Lin et al., 1994]. Standards containing 0, 500, 5,000, 50,000, and 500,000 RNA copies per mL were prepared for testing clinical specimens. For the purpose of method development, standards ranging from 100 copies to 13 million copies per mL also were prepared.

The primary HCV RNA standard was an ACD plasma specimen from a blood donor who was seropositive for HCV. The HCV RNA concentration was determined by the Chiron branched DNA assay [Lau et al., 1993] and by coamplification with the HIV-1 RNA standards described above [Lin et al., 1994]; the results agreed within 10%. Standards for routine use were prepared at concentrations of 1,000, 10,000, 100,000, and 500,000 copies per mL by dilution with ACD plasma specimens that were sero- and PCR-negative for HCV and HIV-1. Other HCV RNA standards, ranging from 500 copies per mL to 21 million copies per mL, were prepared for the purpose of method development. They were calibrated against the primary standard. All standards were aliquoted and stored at −180°C.

Sample Preparation With Spin Columns

The kit employed (QIAamp® HCV Kit, QIAGEN Inc., Chatsworth, CA) included spin columns, collection tubes, virus lysis buffer, wash buffer, carrier RNA (poly A), and diethyl pyrocarbonate (DEPC)-treated water. Additional amounts of DEPC-treated water and extra collection tubes were required (2.0 mL polypropylene microtubes without closure, cat. no. 72.708; Sarstedt, Inc., Newton, NC). Following the manufacturer's directions, five centrifugation steps were performed prior to elution. Two centrifugations were required to load the sample on the column, which was washed by centrifugation with two portions of wash buffer, and then centrifuged without further addition of fluid to remove the last traces of buffer. Clean collection tubes were used for each of these five centrifugation steps. To obtain a tight seal between the spin column and the sample tube during the elution step, we used 2.0 mL boilproof microtubes (cat. no. MH-820; Phenix Research Products, Hayward, CA) that were selected for their fit to

TABLE I. Efficiency of HIV-1 RNA Elution From the Spin Column*

HIV-1 RNA standard (copies/mL)	HIV-1 RNA recovered in the first elution step[a]		
	Percentage (mean ± SD)	Signal-to-noise ratio[b]	Number of copies[c]
650	96 ± 4.6	1.91	87
1,300	98 ± 2.8	3.24	178
13,000	98 ± 1.4	43.8	1,780
130,000	95 ± 1.0	243	17,290
1,000,000	91 ± 2.8	449	126,800

*One hundred forty–microliter samples of ACD plasma with known HIV-1 RNA copy numbers were applied to spin columns as described in Materials and Methods. To determine the efficiency of elution, the spin columns were eluted twice. The presence of HIV-1 RNA in each eluate was determined by means of RT-PCR and chemiluminescent assays.
[a]The total amount of RNA recovered in the two elutions was based on the chemiluminescence (expressed as relative light units, RLU) observed in the first and second eluates.
[b]"Signal" was the RLU obtained with the sample. "Noise" was defined as the mean RLU with zero copy number samples.
[c]The number of HIV-1 RNA copies present in the first eluate was the product of the concentration of the standard, the sample size, and the mean percentage recovery.

the spin columns. The caps of these tubes were cut off at the hinge, and the tubes were autoclaved.

Spin column purification was carried out conveniently in batches of 12 columns. In brief, a 140-μL sample of standard, ACD plasma, heparin plasma, or serum was added to 560 μL of freshly made lysis buffer-carrier reagent and vortexed. After standing at room temperature (20–25°C) for 10 min, 560 μL absolute ethanol was added, and the tube was vortexed. Half of the mixture (630 μL) was transferred to a spin column and centrifuged; this step was repeated using the same spin column. Except where otherwise specified, all centrifugations were carried out in a table-top centrifuge with a horizontal rotor at 8,000*g* for 1 min at 20–25°C. After adding 500 μL of wash buffer to each column, the spin column was centrifuged; this step was repeated. After the second wash, the column was centrifuged again at 13,000*g* for 2 min. Elution was performed as follows. Sterile boilproof tubes were preheated in a dry bath or incubator at 45°C for at least 3 min. The spin columns were firmly inserted into the tubes and the caps were opened. Exactly 160 μL of DEPC-treated water (preheated to 45°C) was added to each tube. The cap was closed, and the whole assembly was held in the dry bath for 5 min before being removed for centrifugation. The eluate was mixed by gentle shaking, taking care not to leave drops on the sides of the tube.

Spin column eluates prepared from clinical specimens were diluted with DEPC-treated water in sterile tubes and mixed by brief vortexing. For HIV RNA, 25 μL of the eluate was diluted with 100 μL water. For HCV RNA, a 1:100 dilution was made with 10 μL eluate. Eluates prepared from the standards and the in-house positive control were not diluted.

Heparinase I Treatment

Sample preparation of heparin plasma was identical with that for serum or ACD plasma, except that the eluates were treated with heparinase I. Heparinase I (Sigma Chemical Co., St. Louis, MO) was dissolved and brought to a concentration of 0.65 units/μL with a sterile solution of glycerol mixed with an equal volume of 0.01 M Tris-HCl (pH 7.6 at 25°C). Placental ribonuclease inhibitor (30–35 units/μL) was obtained from Pharmacia Biotech (Newark, NJ). Heparinase buffer was composed of a sterile solution of 0.065 M Tris-HCl (pH 7.6 at 25°C), 0.013 M calcium chloride, and 0.01 M dithiothreitol.Heparinase reagent was prepared by mixing 100 μL of heparinase buffer with 10 μL (6.5 units) of the heparinase I solution and about 20 μL (650 units) of ribonuclease inhibitor. Exactly 11 μL of heparinase reagent was added to 132 μL of the spin column eluate. After 2 hr at 20–25°C, the samples were used for RT-PCR. To correct for the change in volume introduced by the addition of heparinase reagent a dilution factor of 1.08 was applied.

RT-PCR

A 50-μL sample of the eluate was mixed with 50 μL PCR reagent. The reagent was composed of 0.02 M Tris-HCl (pH 8.3), 0.10 M KCl, 7.4 mM $MgCl_2$ for HIV RNA (or 5 mM $MgCl_2$ for HCV RNA), 0.4 mM of each dNTP, 1 μM each of each primer, 1.5 mM dithiothreitol, and (per μL) 0.5 units ribonuclease inhibitor, 0.05 units Taq DNA polymerase, and 0.4 units murine Moloney leukemia virus reverse transcriptase. The Perkin Elmer model 9600 thermal cycler was employed with heating at 42°C for 1 hr, followed by 22–24 cycles of heating at 94°C for 30 sec for either assay, 55°C for 30 sec for HIV-1 RNA (or 59°C for 30 sec for HCV RNA), and 60°C for 2 min for HIV-1 RNA (or 72°C for 2 min for HCV RNA). The number of PCR cycles was optimized for each lot of streptavidin-coated microtiter plates with the objective of obtaining a standard curve that was almost linear. Target values for signal-to-noise ratios (defined under Table I) were about 2 for the lowest positive standard in each assay and 400 for the 500,000 copy number standard. Amplified samples were vortexed briefly before carrying out the detection procedure.

Chemiluminescent Detection of PCR Products

Distilled water was used to make up the buffer and to wash the plates. Five microliters of the amplified sample was mixed with sample diluent, alkali-dena-

TABLE II. Lower Limits of Detection for the HIV-1 RNA and HCV RNA Assays*

Viral RNA (copies/mL)	n	Signal-to-noise ratio, mean ± SD	Number of positive results (%)[a]
HIV-1 RNA			
100	8	1.36 ± 0.24	0/8 (0)
300	12	1.83 ± 0.21	3/12 (25)
500	12	2.41 ± 0.42	11/12 (92)
800	8	3.19 ± 0.82	8/8 (100)
HCV RNA			
500	6	1.61 ± 0.66	2/6 (33)
1,000	9	2.31 ± 0.31	8/9 (89)
2,000	6	4.97 ± 1.41	6/6 (100)

*Samples of zero or low copy number standards were processed through spin columns using the standard procedure given in Materials and Methods.
[a]Results were considered positive if the RLU of the test sample was above the cut-off level, which was defined as mean RLU of the zero copy number samples plus 3 times its SD.

tured at 20–25°C, and hybridized to the RNA probe at 65°C for 30 min. The samples were transferred to streptavidin-coated wells of a microtiter plate, which was shaken at 400 rpm for 30 min at 20–25°C. After removal of the unbound fraction the wells were washed for 30 sec with a single portion of water. Enzyme-conjugated antibody was added, and the plate was shaken again for 1 hr for HIV-1 RNA (or 30 min for HCV RNA). The plate was washed five times with buffer, then once with water, using an automatic washer/aspirator (Dynatech Laboratories, Inc., Chantilly, VA). After addition of LumiPhos® substrate the plate was incubated at 37°C for 1 hr for HIV RNA assays (or 10 min for HCV RNA). Chemiluminescence was read at 37°C, 5–20 min after placing the plate on the prewarmed carrier of the Microlite Model ML2200 Luminometer (Dynatech Laboratories). The chemiluminescence of the standards, expressed as relative light units (RLU), was plotted as a function of copy number on log-log paper to obtain the standard curve. The copy numbers of the samples were obtained directly from the standard curve, and dilution factors were applied, as appropriate.

RESULTS AND DISCUSSION

Efficiency of the Spin Column

Table I summarizes experiments in which the performance of the spin column for the purpose of HIV-1 RNA purification was evaluated. HIV-1 RNA standards ranging from 650 copies to 1 million copies per mL were applied to the columns. Two elutions were carried out to determine the efficiency of binding, and samples of both eluates were assayed for HIV-1 RNA. Recoveries of HIV-1 RNA in the first elution step averaged 96% with no statistically significant differences in the percentage recoveries from samples with low or high copy numbers (Student's *t*-test). The number of copies that could be bound to and eluted from the spin column was estimated at about 90 to over 10^5. The efficiency of HIV-1 RNA binding to and elution from the spin columns was shown by the chemiluminescent assays performed on the eluates. When plotted on a log-log scale, the signal-to-noise ratios fell on a smooth curve. Linear regression analysis showed direct proportionality extending from 650 to over 13,000 copies per mL.

With regard to the assay of clinical specimens, it was unnecessary to aim for 100% recovery over the given range of HIV-1 RNA concentrations because both standards and clinical specimens were processed in parallel. Thus, in the routine protocol the spin columns were loaded and washed in five centrifugation steps, and eluted once with DEPC-treated water, as described in Materials and Methods.

Lower Limit of Detection

The lower limit of detection of the HIV-1 RNA assay was considered to be 500 copies per mL (Table II). Typically, the result obtained with zero copy number samples was 0.3 RLU with a SD of <0.1 and was indistinguishable from the results obtained with reagent blanks that were likewise taken through the steps of amplification and chemiluminescent detection, or those obtained on samples of water subjected only to the chemiluminescent assay procedure. The cut-off level was set at the mean RLU value of zero copy number samples plus 3 SD, and samples yielding RLU values above this level were considered positive. Because the positivity rates for HIV-1 RNA standards with 100, 300, 500, and 800 copy numbers were, respectively, 0, 25, 92, and 100%, we placed the lower limit of detection at 500 copies HIV-1 RNA per mL.

The empirical determinations of the positivity rates for RT-PCR were consistent with the Poisson law [Strike, 1991]. The Poisson distribution predicts that with an average of two, three, four, or five RNA copies per sample, the probabilities of obtaining a negative result (i.e., of testing a sample with no RNA copies) would be, respectively, 14, 5, 2, and 0.7%. The samples that were used for amplification and detection represented about 44 μL of the standard. Thus, a 44-μL sample of the 500 copy number standard would contain, on the average, 22 RNA copies. with an estimated 10–20% efficiency in the reverse transcription step [Lin et al., 1996], the number of cDNA copies produced would be, on the average, two to four copies. It could be expected, then, that the positivity rate would be in the range of 86 to 98%. The observed positivity rate for the 500 copy number standard fell within this range.

Table II also shows that the lower limit of detection

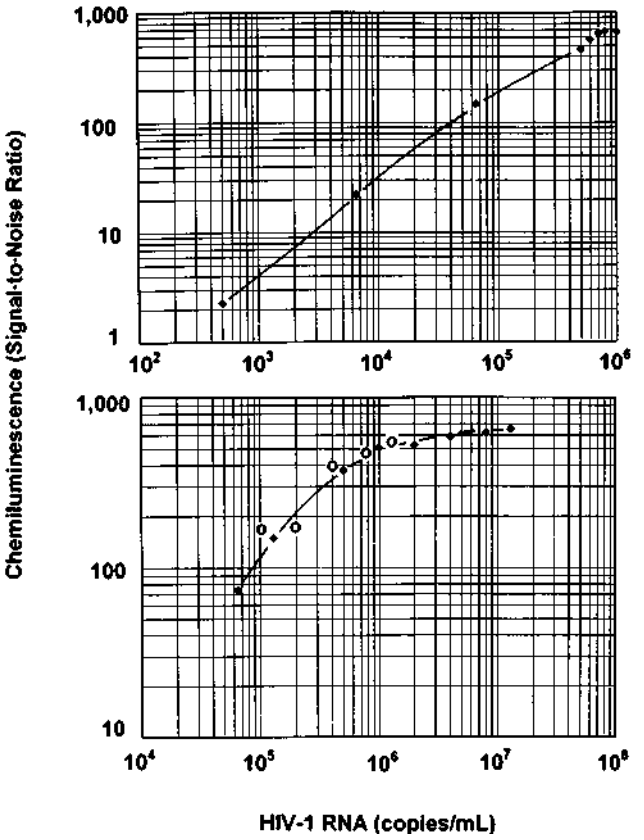

Fig. 1. The upper limit of chemiluminescent assay for detection of HIV-1 RNA PCR products. **Top:** the upper limit was 500,000 copies per mL. The chemiluminescent signals obtained with samples of the 600,000 copy number standard were consistently higher than those obtained with the 500,000 copy number standard, while those obtained with 700,000, 800,000 and 1 million copy number standards were nearly the same. **Bottom:** The plateau effect observed at high copy numbers was attributable to the chemiluminescent assay. Solid symbols, RLU values obtained with samples amplified from HIV-1 RNA standards ranging from 65,000 to 13 million copies per mL; open symbols, RLU obtained on tenfold dilutions of the same amplified samples of the five highest standards (1, 2, 4, 8, and 13 million copies per mL).

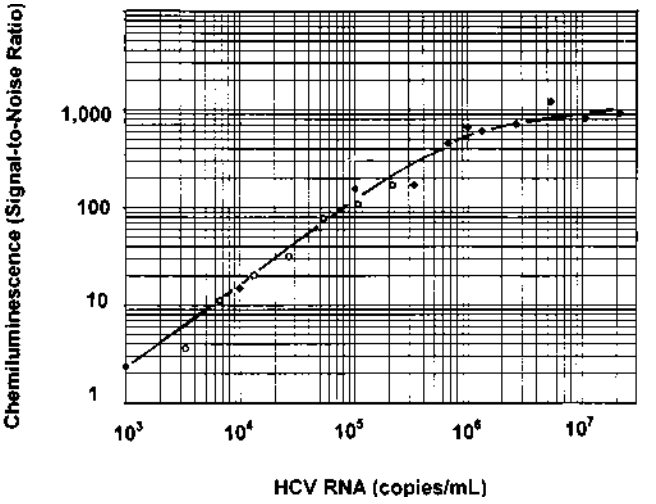

Fig. 2. The upper limit of chemiluminescent detection of HCV RNA PCR products. Samples of a twofold dilution series (330,000 to 21 million copies per mL), together with four HCV RNA standards at 1,000, 10,000, 100,000, and 1 million copies per mL, were processed through spin columns. Reverse transcription-PCR was carried out on the spin column eluate with and without dilution. Solid symbols, RLU values obtained on samples amplified without dilution; open symbols, RLU obtained on samples that had been diluted 1:100 from the eluates prepared from the dilution series; the concentrations represented by the 100-fold dilutions ranged from 3,300 to 210,000 copies/mL.

of the HCV RNA assay was higher than that for HIV-1 RNA. A concentration of 1,000 HCV RNA copies per mL was required to achieve a positivity rate of about 90%. In the HCV RNA assay the RLU of the zero copy number samples were consistently higher than those observed in the HIV-1 RNA assay, introducing a greater degree of imprecision. Compared to HIV-1 RNA, about twice as many copies of HCV RNA achieved a signal-to-noise ratio of about 2.

Upper Limit of Detection

The upper limit of detection in the HIV-1 RNA assay was 500,000 copies per mL (Fig. 1, top panel). The RLU obtained on the 600,000 copy number standard was consistently and significantly higher than that obtained on the 500,000 copy number standard. These observations established the upper limit of detection of the chemiluminescent assay as 500,000 copies per mL and suggested that the standard curve could be safely extrapolated to 550,000 copies per mL. Above 600,000 copies per mL, the RLU values tended to overlap each other. Thus, standards with 700,000, 800,000, 1 million copies per mL, and above produced chemiluminescent signals of similar intensities. In this range of HIV-1 concentrations, the standard curve appeared to reach a plateau.

The reason underlying the plateau effect was investigated using standards ranging from 1 to 13 million copies per mL (Fig. 1, bottom panel). The mean RLU increased only by about 25% over this range. The plateau could have resulted from a limiting factor in the chemiluminescent assay such as the amount of antibody or a limiting component of the RT-PCR assay such as the number of primer molecules used. Chemiluminescent assays were carried out on amplified samples of 1, 2, 4, 8, and 13 million copy number standards and on tenfold dilutions of the same samples. The RLU of the diluted samples fell on a curve that coincided with that obtained with undiluted standards in the range 65,000 to 2 million copies per mL. Had the limiting factor been present in the RT-PCR step, the tenfold diluted samples would be expected to produce RLU values that fell on a straight line parallel to the x-axis, but such was not the case. The RLU values of the diluted samples showed gradations consistent with different HIV-1 RNA copy numbers. This proved that the limiting factor was associated with the chemiluminescent assay.

Similar experiments were carried out with HCV RNA. Figure 2 shows that the standard curve was almost linear to about 600,000 copies per mL. The upper limit of chemiluminescent detection of PCR products in

TABLE III. Application of the Spin Column to the Assay of HIV-1 RNA in Different Cell-Free Blood Specimens*

Subject	HIV-1 RNA (copies per mL)		
	ACD plasma	Heparin plasma[a]	Serum
1	1,200	1,000	870
2	1,400	1,400	500
3	6,100	5,200	2,000
4	6,800	5,900	2,100
5	8,200	14,000	8,200
6	9,500	9,400	3,500
7	12,500	13,000	7,200
8	40,000	44,000	34,000
9	48,000	49,000	50,000
10	66,000	95,000	18,500

*Samples of the blood specimen drawn from each subject were collected in serum-separating tubes and Vacutainer tubes containing acid-citrate dextrose (ACD) or heparin anticoagulant. Serum and plasma specimens were processed using the spin column procedure described in Materials and Methods.
[a]Heparinized plasma samples were processed by the standard spin column procedure, followed by treatment of the eluate with heprinase I as given in Materials and Methods.

the HCV RNA assay was conservatively set at 500,000 copies per mL. The data presented in Figures 1 and 2 showed, further, how the dynamic ranges of the assays for either HIV-1 RNA or HCV RNA could be expanded. By including samples of a 1:5 dilution of the spin column eluate prepared from clinical HIV-1 RNA specimens (or a 1:100 dilution of HCV RNA eluate) in PCR-based amplification, the dynamic ranges of the two assays were extended to 2.5 million HIV-1 RNA copies per mL and to 50 million HCV RNA copies per mL.

Precision and Accuracy

The overall imprecision of the recommended protocol was evaluated in two stages because the changes involved both sample preparation and detection methods. The following data were obtained on the HIV-1 RNA assay. To assess the accuracy of the chemiluminescent detection method, we compared the results against those obtained by the colorimetric assay described previously [Lin et al., 1996]. HIV-1 RNA was extracted with the guanidinium isothiocyanate-phenol-chloroform (GTPC) procedure from 11 different clinical specimens and from HIV-1 RNA standards. Different samples of each preparation were subjected to reverse transcription and amplification with the number of PCR cycles that was appropriate for each detection method, and the copy numbers were determined. The observed concentrations ranged from 10^3 to 10^5 copies per mL. Correlation between the results obtained on the 11 clinical specimens by the two detection methods was given by r = 0.98, and linear regression of y (chemiluminescent detection) on x (colorimetric detection) was $y = 0.80x + 1907$. We then compared the imprecision of the chemiluminescent assay used on samples prepared with different extraction methods that employed either GTPC or spin columns. With GTPC, between-run coefficients of variation (CV) found in two specimens with copy numbers of 6,900 and 58,000 were, respectively, 25.7% and 14.4% (n = 5). Using spin columns for RNA purification, between-run imprecision was 23% at 16,000 copies per mL (n = 14).

HIV-1 RNA concentrations obtained in the 1:5 dilution versus that in the undiluted samples were concordant. In 38 specimens with mean HIV-1 RNA concentrations ranging from 2,700 copies per mL to 350,000 copies per mL that were amplified neat and at a 1:5 dilution, the differences between 38 pairs of results were not statistically significant ($P = .41$, Student's paired t-test). On the average, the result on the undiluted sample and the result on the 1:5 dilution (multiplied by the dilution factor) agreed within 4%.

To assess the accuracy of the recommended HCV RNA assay, we compared the results obtained on 11 clinical specimens against values obtained on the same specimens by the bDNA method. HCV RNA concentrations in these specimens ranged from 1.1 to 17 million by the recommended method, or from 1 to 28.6 million by the bDNA method. Correlation between the two sets of results was good (r = 0.91), and differences within each pair were not statistically significant ($P = .065$).

Application of the Spin Column to Different Specimen Types

Heparin interferes with both reverse transcription and PCR [Izraeli et al., 1991; Beutler et al., 1990]. Silica has been used to separate DNA and RNA from heparinized specimens [Boom et al., 1990]. We tested the application of silica-based spin columns to plasma containing ACD or heparin anticoagulant, and to serum. Initially, all heparinized plasma specimens were processed through spin columns in the manner specified in Materials and Methods for serum or ACD plasma, amplified, and assayed for PCR products. The chemiluminescence signals in the heparinized plasma preparations were the same as those given by reagent blanks. The application of two extra washes, making a total of four, produced the same result.

It was necessary to degrade the heparin in the spin column eluate with heparinase before carrying out RT-PCR, using the procedure detailed in Materials and Methods. Table III shows the comparison of HIV-1 RNA copy numbers in different types of specimens processed

TABLE IV. Estimates of HIV-1 RNA to HIV-1 Proviral DNA Ratios in Blood

Subject	HIV-1 RNA (copies per mL plasma)[a]	HIV-1 DNA copies		RNA:DNA ratio in whole blood[c]
		Per 10^6 PBMC[b]	Per mL blood	
1	1,500	630	1,512	0.6
2	1,700	250	425	2.4
3	2,500	150	390	3.8
4	5,700	350	630	5.4
5	8,800	680	1,836	2.9
6	11,500	1,600	7,840	0.9
7	18,200	930	1,395	7.8
8	22,800	390	1,950	7.0
9	39,200	120	132	178.2
10	50,000	1,200	852	35.2
11	66,400	290	870	45.8
12	137,200	1,500	1,650	49.9
13	139,900	850	697	120.4
14	278,700	920	754	221.8
15	366,300	990	1,307	168.2

[a]Median value based on assays at ten sites [Lin et al., 1994].
[b]PBMC, peripheral blood mononuclear cells. PBMC counts ranged from 0.7 million to 5.0 million per mL.
[c]Assuming a hematocrit of 40%, the ratio was 0.6 times the RNA copies per mL plasma divided by the DNA copies per mL blood.

through the spin columns. Within-subject comparisons of copy numbers were carried out (paired Student's *t*-test). Copy numbers obtained in ACD plasma samples were not significantly different from those in heparinase-treated samples prepared from heparin plasma ($P = .20$), but copy numbers in serum were significantly different from those obtained in ACD plasma ($P = .002$) or heparinase-treated heparin plasma samples ($P = .008$). On the average, copy numbers obtained on serum samples were 40% lower than those in ACD plasma samples. These results were in line with reports on the degradation of hepatitis C virus RNA in serum as compared to ACD plasma [Busch et al., 1992] and on HIV-1 RNA copy numbers obtained in different specimen types using the branched DNA assay [Holodniy et al., 1995].

The possibility that the endogenous heparin normally present in blood plasma might interfere significantly with the HIV-1 RNA was excluded. Heparinase treatment of five ACD plasma showed no significant differences in HIV-1 RNA copy number in the treated sample compared to that obtained in an untreated sample of the same ACD plasma ($P = .53$).

HIV-1 RNA to DNA Ratios in Whole Blood

Because the spin column could bind both DNA and RNA, the extent of HIV-1 proviral DNA contamination in HIV-1 RNA preparations was evaluated. HIV-1 DNA could be present in cell-free plasma or serum because of lysis of infected cells occurring in vivo, or as the result of prolonged standing in the presence of blood cells after collection of the specimen. Table IV gives the estimate of HIV-1 RNA to HIV-1 DNA ratios in whole blood, based on data collected in 15 patients studied previously [Lin et al., 1994]. With the exception of subjects 1 and 6, RNA was the predominant HIV nucleic acid in the blood specimens. The ratio of HIV-1 RNA to DNA increased with higher RNA concentrations, which was in part attributable to the decrease in the number of HIV-1-infected CD4 cells in more severe viremia.

Experiments were carried out to determine the extent to which HIV-1 proviral DNA could affect the assay of HIV-1 RNA. HIV-1 RNA-positive plasma samples were spiked with 8E5/LAV cells which contain one copy of proviral DNA per cell [Folks et al., 1986]. After RT-PCR amplification, spuriously higher HIV-1 RNA concentrations were observed in samples that contained about 20 proviral DNA copies per amplification. We then tested specifically for HIV-1 DNA in plasma by omitting reverse transcriptase from the assay. The plasma specimens listed in Table IV were available, with the exception of samples from subjects 5, 9, and 15. No HIV-1 DNA was detected in any of the 12 plasma samples. The plasma had been separated from blood within 6 hr of collection and stored at −180°C [Lin et al., 1994]. Prompt separation of plasma from blood cells could eliminate proviral HIV-1 DNA as a source of interference in the PCR-based assay of HIV-1 RNA.

CONCLUDING REMARKS

Although the components of the procedures described in this report are not totally new, their use in combination resulted in several advantages over some published methods. The use of spin columns bypassed the requirements for high-speed centrifugation, binding to silica particles in suspension, or alcohol precipitation that have been variously employed in HIV-1 RNA purification procedures [Boom et al., 1990; Winters et al., 1993; Lin et al., 1994].

Chemiluminescent detection of PCR products enabled a broad dynamic range to be achieved with the use of a single dilution made prior to amplification. It also enabled sensitive assays to be performed on small blood samples. The lower limit of detection for HIV-1 RNA using a single sample of 140 μL was 500 copies per mL, which was comparable to the sensitivity of

about 200 copies/mL reported to be achievable with 200-μL samples using colorimetric detection [Mulder et al., 1994].

The microtiter plate luminometer was an essential piece of equipment for the recommended procedures, whereas the automatic plate washer was convenient but not essential. The cost of the luminometer may be offset by savings in other areas. The cost of reagents and other comsumables including plasticware for batch testing of 20 clinical specimens came to about $30 per specimen. Since spin column-chemiluminescent assay procedures were relatively easy to carry out and not technically demanding, there would be further savings in time and labor.

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